THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 50, pp. 42269–42277, December 7, 2012

Enforced Expression of Roquin Protein in T Cells Exacerbates the Incidence and Severity of Experimental Arthritis*

Received for publication, April 23, 2012, and in revised form, October 6, 2012 Published, JBC Papers in Press, October 12, 2012, DOI 10.1074/jbc.M112.374835

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Background: Roquin regulates inflammation in autoimmune disease.
Results: Roquin overexpression promoted the induction of collagen-induced arthritis (CIA).
Conclusion: Enforced Roquin expression was produced in mice and used to demonstrate the importance of inflammation and CIA development in experimental arthritis.
Significance: Roquin overexpression may induce inflammation in some autoimmune diseases.

To investigate the role of Roquin, a RING-type ubiquitin ligase family member, we used transgenic mice with enforced Roquin expression in T cells, with collagen-induced arthritis (CIA). Wild-type (WT) and Roquin transgenic (Tg) mice were immunized with bovine type II collagen (CII). Arthritis severity was evaluated by clinical score; histopathologic CIA severity; proinflammatory and anti-inflammatory cytokine levels; anti-CII antibody levels; and populations of Th1, Th2, germinal center B cells, and follicular helper T cells in CIA. T cell proliferation *in vitro* and cytokine levels were determined to assess the response to CII. Roquin Tg mice developed more severe CIA and joint destruction compared with WT mice. Production of TNF-α, IFN-γ, IL-6, and pathogenic anti-collagen CII-specific IgG and IgG2a antibodies was increased in Roquin Tg mice. In addition, *in vitro* T cell assays showed increased proliferation and proinflammatory cytokine production in response to CII as a result of enforced Roquin expression in T cells. Furthermore, the Th1/Th2 balance was altered by an increased Th1 and decreased Th2 population. These findings suggest that overexpression of Roquin exacerbates the development of CIA and that enforced expression of Roquin in T cells may promote autoimmune diseases such as CIA.

Rheumatoid arthritis (RA)[4] is a severe inflammatory disease with autoimmune features and an intricate genetic element. RA is characterized by synovial hyperplasia, chronic inflammation, and infiltration of lymphocytes; it is driven by diverse cellular and humoral autoimmune responses and results in the destruction of cartilage and bone (1, 2). Autoimmune arthritis is induced in the collagen-induced arthritis (CIA) mouse model via immunization with an emulsion of complete Freund's adjuvant and type II collagen (CII) (3). Considerable data implicate CII-reactive $CD4^+$ cells as the primary mediators of disease induction and autoantibody production by B cells as a major immune mechanism leading to a localized chronic inflammatory response (4–6). The CIA model has been used extensively to explain various mechanisms related to human RA and to identity potential targets for therapy. Therefore, we used the CIA model to study gene function in autoimmune diseases such as RA.

T cell activation and differentiation are regulated not only by T cell receptor recognition of the antigen-major histocompatibility complex (MHC) but also by interactions between many co-stimulatory molecules (7). Although the interactions among various immune cells are complex, optimal responses are induced by controlled and coordinated expression of T cell co-stimulatory receptors. CD28, the first co-stimulatory molecule to be identified and arguably the most important in naive T cell activation, binds to B7.1 (CD80) and B7.2 (CD86), which are expressed on the surface of professional antigen-presenting cells. CD28 co-stimulation enhances T cell proliferation, cytokine production, and survival (8–10). Studies using T cells derived from CD28-deficient mice demonstrated its role in T cell activation (11). Consistent with this, CD28 knock-out mice with the DBA/1 background are resistant to CIA (12). Inducible T cell co-stimulator (ICOS), another member of the CD28 family, is not expressed by naive T helper (Th) cells but is induced

* This work was supported by Next-Generation BioGreen21 Program Grant PJ 008050 from the Rural Development Administration, Republic of Korea and by National Research Foundation of Korea Grant 2012-0000640 funded by the Korea government (Ministry of Education, Science and Technology) and Woo Jang-Choon (PJ007849) projects from the Rural Development Administration, Republic of Korea.
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[4] The abbreviations used are: RA, rheumatoid arthritis; CII, type II collagen; CIA, collagen-induced arthritis; ICOS, inducible T cell co-stimulator; Th, T helper; Tfh, follicular helper T; PerCP, *peridinin-chlorophyll protein complex; PE, phycoerythrin; APC,* allophycocyanin; Tbet, T-box *expressed in T cells; Tg, transgenic.*

after T cell activation (13, 14). Recently, several groups have constructed and analyzed ICOS-deficient mice (15–17). T cells derived from ICOS-deficient mice exhibit reduced proliferation and IL-2 production, indicating a role for ICOS in T cell activation (15, 17), and are resistant to CIA (7).

Roquin was recently identified as a RING-type ubiquitin ligase family member. Roquin mRNA is ubiquitously expressed, and Roquin protein has been predicted to be intracellular. Roquin contains an N-terminal RING-1 zinc finger at residues 14–53 based on consensus sequences with the E3 ubiquitin ligase family. The C terminus of the RING finger is a highly conserved novel protein domain known as ROQ, and a zinc finger domain of the CCCH type that conforms precisely to the RNA-binding zinc finger occurs in the center of Roquin. The C terminus of the CCCH domain is a proline-rich region representing potential sites for binding the Src homology 3 (SH3) domains of interacting proteins (19, 20).

Notably, one report identified a novel repressor of ICOS, Roquin, which is the defective gene in sanroque mice (20). san/san mice have a point mutation in Roquin and are highly susceptible to autoimmunity. $CD4^+$ T cells from san/san mice express higher levels of ICOS than do those of wild-type (WT) controls. Particularly striking are the accumulation of follicular helper T (Tfh) cells and the increased size of germinal centers. However, in our previous study, Roquin overexpression induced up-regulation of CD28 expression as well as down-regulation of ICOS during T cell activation by anti-CD3/CD28. Secretion of IL-2 and proinflammatory cytokines was increased by enforced expression of Roquin in T cell activation (21). Thus, decreased ICOS expression may repress Th cell function and prevent pathogenic immune responses. However, conflicting data on the role of ICOS in allograft survival have been reported, and ICOS blockade can exacerbate or reduce experimental autoimmune encephalomyelitis severity depending upon the treatment approach (15, 22). Moreover, increased CD28 may regulate T cell activation and lead to an autoimmune response.

To our knowledge, there are no reports regarding the function of Roquin in RA in the literature. Therefore, we used Roquin-overexpressing mice to investigate the role of Roquin in the CIA mouse as a model of autoimmune disease. The results indicate that Roquin overexpression promoted the induction of CIA even when reinforced immunization protocols were applied. Immune cell populations, including Th cells, Tfh cells, and germinal center B cells, were unaffected by Roquin overexpression in non-immunized mice. However, Th1/Th2 balance was affected by Roquin overexpression in the CIA model. Therefore, these findings have important implications for our understanding of the role of Roquin in T cells in autoimmune diseases such as RA.

EXPERIMENTAL PROCEDURES

Mice—Roquin transgenic mice were generated as described previously (21). Six generations of Roquin transgenic mice were backcrossed onto the DBA/1 background. All mice used in these experiments were 8–12 weeks old. All animals were raised and kept under specific pathogen-free conditions.

Induction of Collagen-induced Arthritis—Arthritis was induced and scored in male WT and Roquin transgenic mice by immunization with bovine CII (Chondrex). Bovine CII was dissolved in cold 0.01 M acetic acid at 2.5 mg/ml. Mice were immunized by intradermal injection with 100 μg of CII emulsified in complete Freund's adjuvant. On day 21, the mice received a booster immunization with the same amount of CII in incomplete Freund's adjuvant into the left footpad. The incidence and severity of arthritis were evaluated by visual inspection of the paws. Each limb, except the hind foot that received the booster immunization, was assessed on a 0–4-point scale. The severity of arthritis in mice was scored in individual mice in a double blind manner with each paw assigned a separate clinical score as follows: 0, no evidence of erythema or swelling; 1, erythema and mild swelling confined to the ankle joint and toes; 2, erythema and mild swelling extending from the ankle to the midfoot; 3, erythema and severe swelling extending from the ankle to the metatarsal joints; and 4, ankle deformity with joint swelling.

Histological Analysis—On day 45, the mice were killed for histological analysis. Three paws and ankles, with the exception of the hind foot that received the booster immunization, were fixed with 4% paraformaldehyde for 2 days, decalcified in 30% citrate-buffered formic acid at 4 °C, dehydrated in a graded series of ethanol and xylene, and embedded in paraffin. Thin sections (3 μm) were stained with hematoxylin and eosin. Histological changes such as degree of synovial hyperplasia, inflammation, and pannus formation in the joints were scored on a scale from 0 to 3 as follows: 0, absent; 1, weak; 2, moderate; and 3, severe. The maximum possible score per mouse was 9.

Western Blot Analysis—Spleens were prepared using lysis buffer (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.25% Triton X-100, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 10 μg/ml leupeptin, and 2 μM phenylmethylsulfonyl fluoride), and the resulting lysates were cleared by centrifugation. The protein concentrations of the clarified supernatants were determined using a Bradford protein assay (Bio-Rad) with bovine serum albumin as a standard. Whole tissue lysates were fractionated by Tris-glycine-buffered 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes followed by incubation with an antibody to HA (Roche Applied Science) and β-actin (Santa Cruz Biotechnology) overnight at 4 °C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Immunoreactivity was determined using an enhanced chemiluminescence detection system (GE Healthcare). Antibodies against phospho-AKT (Ser-473), AKT, phospho-ERK (Thr-202/Tyr-204), ERK, phospho-JNK (Thr-183/Tyr-185), JNK, phospho-IκBα (Ser-32/36), and IκBα were obtained from Cell Signaling Technology.

Flow Cytometric Analysis—Single cell suspensions were prepared from the lymph nodes and spleens of mice and treated with red blood cell lysing solution (0.15 M NH_4Cl and 0.1 mM Na_2EDTA) for 5 min at 4 °C to eliminate erythrocytes. $CD4^+$ T cells were positively isolated using anti-CD4 monoclonal antibody-coupled magnetic cell-sorting microbeads (Miltenyi Biotech) to more than 95% purity (as analyzed by flow cytometric

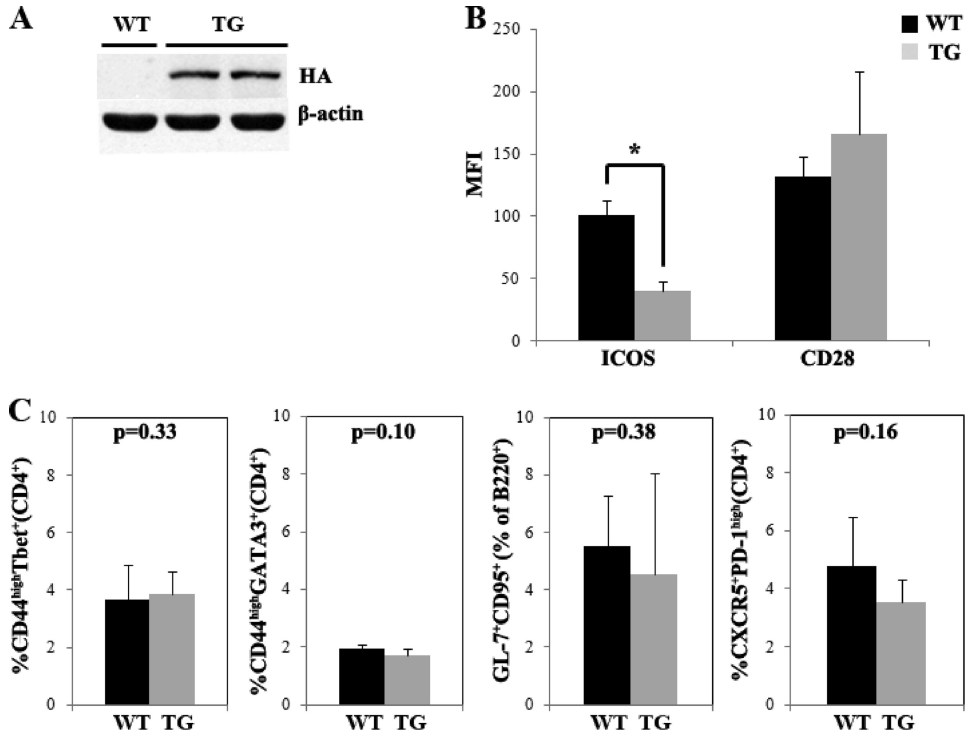

FIGURE 1. **Generation of Roquin-overexpressing transgenic mice and detection of cell populations in WT and Tg mice.** *A*, Western immunoblot analysis using HA antibody to detect Roquin expression in transgenic mice. *B*, the populations of ICOS- and CD28-positive cells among $CD4^+$ T cells from naive DBA/1 and Roquin Tg mice were examined by flow cytometry. Mean fluorescence intensity (*MFI*) values were measured and compared with those of WT mice. *Error bars* indicate mean ± S.E. of triplicates. *, $p < 0.05$. *C*, representative flow cytometric graphical analysis of $Tbet^+CD44^{high}CD4^+$, $GATA3^+CD44^{high}CD4^+$, $B220^+GL\text{-}7^+CD95^+$, and $PD\text{-}1^{high}CXCR5^+CD4^+$ cells in WT and Roquin transgenic mice. *Black bars*, wild-type (WT) mice; *gray bars*, Roquin Tg mice. Data are representative of two independent experiments ($n = 4$ per group). *p* values are indicated on the graphs.

staining with anti-CD4 and anti-CD8 antibodies). Cells were stained with fluorochrome-conjugated anti-mouse Tbet-PerCP Cy5.5, GATA3-AF647, CD44-FITC, CD44-PE, CD44-APC, CD4-PerCP, CXCR5-FITC, PD-1-PE, B220-PerCP, GL-7-FITC, CD95-PE, ICOS-PE, and CD28-PE antibodies and analyzed on a FACScan using CellQuest software (BD Biosciences).

Lymphocyte Proliferation and Cytokine Analysis—Spleen and draining lymph node cells were cultured at 2×10^6 cells/ml for up to 96 h in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 2 mM L-glutamine, 10 mM HEPES (pH 7.4), 1 mM sodium pyruvate, 50 units/ml penicillin, and 50 mg/ml streptomycin in a humidified 5% CO_2 atmosphere. Cells were stimulated with graded concentrations of CII. Cell proliferation was quantified using a DNA bromodeoxyuridine (BrdU) incorporation assay (Roche Applied Science). The amount of incorporated BrdU is a measure of the rate of DNA synthesis and thus indirectly of cell proliferation. The cell proliferation kit was used according to the manufacturer's instructions. Supernatants from parallel triplicate cultures were stored at −70 °C until estimation of cytokine content by enzyme-linked immunosorbent assay (ELISA).

Measurement of Anti-CII Antibodies—Serum was collected on days 20 (diluted 1:5000) and 45 (diluted 1:20,000), and anti-type II collagen antibody levels (total IgG, IgG1, and IgG2a) were measured using ELISA kits purchased from Chondrex.

Cytokine Assays—Total interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-6, IL-4, IL-10, and IL-17 levels in supernatants were determined using a commercially available ELISA kit (R&D Systems) according to the manufacturer's recommendations. The IL-21 levels were determined using a commercial ELISA kit (Biolegend).

Statistical Analysis—The results are expressed as the mean ± S.E. of at least three independent experiments. The significance of differences between groups was calculated using a two-tailed Student's *t* test. Differences with a *p* value of less than 0.05 were considered statistically significant.

RESULTS

Generation of Roquin Transgenic Mice and Modulation of Co-stimulatory Expression in $CD4^+$ T Cells in Naive Mice—To generate transgenic mouse lines that expressed high levels of Roquin specifically in T cells, mouse Roquin cDNA was inserted into a vector containing a human CD2 transgene cassette (21). To confirm the expression of the transgene, Western blotting was used to monitor production of the Roquin-HA fusion protein in the spleens of Roquin-overexpressing transgenic (Tg) mice (Fig. 1*A*). Co-stimulatory receptor expression was assessed by FACS analysis. $CD4^+/CD28^+$ or $CD4^+/ICOS^+$ T cell populations from the spleen were isolated using a $CD4^+$ T cell isolation kit. Our data show decreased ICOS expression in the $CD4^+$ T cells of the Roquin Tg mice compared with WT mice. This result is similar to that of a previous study, which demonstrated that Roquin represses ICOS expression (23) but has no effect on CD28 expression in $CD4^+$ T cells (Fig. 1*B*).

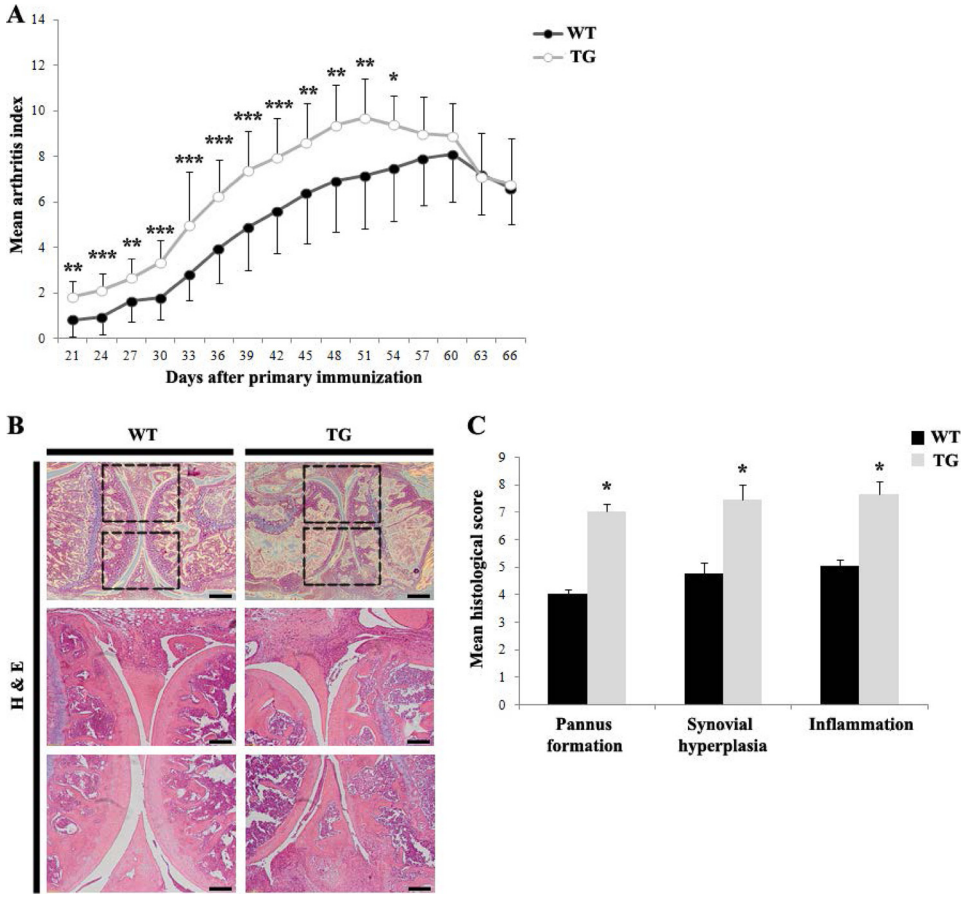

FIGURE 2. **Enforced Roquin expression in T cells exacerbates CIA development as assessed by clinical score and histology.** *A*, WT ($n = 30$) and Roquin Tg ($n = 30$) mice were immunized (day 0) and given a booster immunization (day 21) with CII. The clinical severity of arthritis in each paw, except the hind foot that received the booster immunization, was scored using a semiquantitative scoring system (0–4 scale; maximum total score, 12 per animal). *Solid symbols*, WT; *open symbols*, Roquin Tg mice. *Error bars* indicate mean ± S.E. of all immunized mice of each genotype. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *B*, histological examination of the joints from WT and Roquin Tg mice. Mice were killed on day 45 after primary immunization. All paws and ankles, except the hind foot that received the booster immunization, were harvested from each mouse and stained with hematoxylin and eosin. *Scale bars*, 200 (*upper panel*) and 100 μm (*middle* and *lower panels*). *C*, mean histology scores for the degree of pannus formation, synovial hyperplasia, and inflammation in WT and Roquin Tg mice on day 45. *Black bars*, WT mice; *gray bars*, Roquin Tg mice. *Error bars* indicate mean ± S.E. of all immunized mice of each genotype ($n = 6$). *, $p < 0.05$.

No Effect on the Number of Lymphocytes in the Spleens of Naive Mice—To evaluate the lymphocyte population in naive mice, we analyzed the lymphocyte subsets in the spleen. The percentages of $Tbet^+CD44^{high}CD4^+$ T (Th1) and $GATA3^+CD44^{high}CD4^+$ T (Th2) cells were not altered in the Roquin Tg mice compared with the WT mice ($p = 0.33$ and $p = 0.10$, respectively). Similarly, the percentages of $B220^+GL\text{-}7^+CD95^+$ (germinal center B) and $PD\text{-}1^{high}CXCR5^+CD4^+$ (Tfh) cells were not altered in the Roquin Tg mice (Fig. 1*C*). These results demonstrate that T cells and germinal center B cells developed normally in the spleens of the Roquin Tg mice.

CIA Promotion and Severity in Roquin Tg Mice—To assess whether T cell-specific Roquin expression affects the development of arthritis, we examined experimental CIA in Roquin Tg and WT mice. The clinical arthritis score was significantly higher in the Roquin Tg mice than in the WT mice, and arthritis arose earlier in the Roquin Tg mice relative to the WT mice (Fig. 2*A*). In the chronic state (*i.e.* after 60 days), the clinical scores of the Roquin Tg and WT mice were similar. Moreover, histological analyses of joints obtained 45 days after immunization revealed that joint inflammation and destruction were significantly accelerated in the Roquin Tg mice compared with the WT mice (Fig. 2*B*). Histology scores for the degree of pannus formation, synovial hyperplasia, and inflammation were higher in the Roquin Tg mice than in the WT mice on day 45 (Fig. 2*C*). These results indicate that enforced expression of Roquin in T cells promoted inflammation and the development of CIA.

Induction of Proinflammatory Cytokine Production and Signaling Modulation in Roquin Tg Mice—Because enforced Roquin expression in T cells promoted the development of CIA, we examined whether Roquin regulates the secretion of inflammatory cytokines. We isolated serum from mice at 20 and 45 days after immunization and then assessed the levels of various cytokines by ELISAs. In a previous study, CD28-induced IL-2 secretion was increased by Roquin overexpression *in vitro* (21). To confirm whether IL-2 secretion is increased in autoimmunity as it is *in vivo*, we performed an ELISA on serum collected from CIA mice at 20 and 45 days after immunization. IL-2 secretion was significantly increased in the Roquin Tg mice compared with the WT mice ($p < 0.05$) (Fig. 3*A*). At 20 days after immunization, the secretion of TNF-α, IL-6, and IL-17 was significantly elevated in Roquin Tg mice compared with WT mice. However, the levels of IFN-γ, IL-10, and IL-21 were unchanged. At 45 days after immunization, the levels of

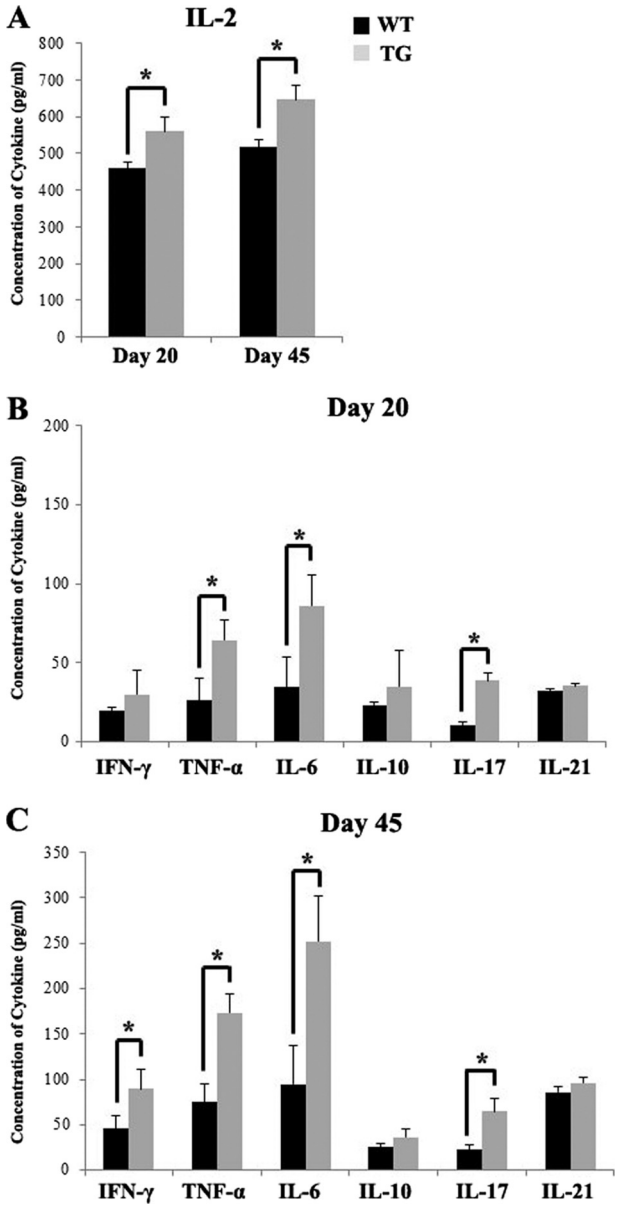

FIGURE 3. **Increased proinflammatory cytokine production by Roquin in CIA mice.** *A*, secretion of IL-2 was detected by ELISA on days 20 and 45 in serum from Roquin Tg and WT mice after immunization. *Error bars* indicate mean ± S.E. of all immunized mice of each genotype ($n = 6$). *, $p < 0.05$. *B* and *C*, serum cytokine concentrations in WT and Roquin Tg mice. Sera were obtained from mice on days 20 and 45, and the levels of IFN-γ, TNF-α, IL-6, IL-10, IL-17, and IL-21 were measured by ELISA. *Black bars*, WT mice; *gray bars*, Roquin Tg mice. *Error bars* indicate mean ± S.E. of all immunized mice of each genotype ($n = 6$). *, $p < 0.05$.

TNF-α, IFN-γ, IL-6, and IL-17 were significantly increased in the Roquin Tg mice compared with WT mice. However, the levels of IL-10 and IL-21 were unchanged (Fig. 3, *B* and *C*). Moreover, downstream signals of CD28, which regulates IL-2 transcription, were detected in the spleens of CIA mice at 45 days after immunization. Phosphorylation of AKT, JNK, and IκBα, but not ERK, was significantly increased in Roquin Tg mice compared with WT mice (Fig. 4). These results indicate that enforced expression of Roquin in T cells increased the production of proinflammatory cytokines and that activation of AKT, JNK, and NF-κB, all downstream of signals CD28, induced IL-2 secretion in Roquin Tg mice.

Alteration of CII-specific IgG Production in Roquin Tg Mice—Because CII-specific IgG levels correlated well with the development of arthritis, we examined CII-specific IgG production in Roquin Tg mice. Serum was isolated from each mouse at 20 and 45 days after immunization. CII-specific total IgG levels measured by ELISA were significantly higher in Roquin Tg mice than in WT mice. At 20 and 45 days after immunization, CII-specific IgG2a, which is associated with Th1 class switching, was higher in Roquin Tg mice, but CII-specific IgG1, which is associated with Th2 class switching, was lower in Roquin Tg mice compared with WT mice (Fig. 5). Thus, enforced expression of Roquin in T cells caused increased production and differential regulation of CII-specific IgG by B cells.

Increased Immune Response in the Spleen and Draining Lymph Nodes—We examined whether Roquin alters the immune response to CII. Spleen and draining lymph node cells were obtained from each group of mice at 20 and 45 days after CII immunization, and their ability to produce IFN-γ, TNF-α, IL-6, IL-10, and IL-17 or to proliferate in response to CII dose-dependently was investigated. After *in vitro* antigenic stimulation with CII, proliferation of spleen and draining lymph node cells was higher in Roquin Tg mice than in WT mice at 20 and 45 days after immunization in a dose-dependent manner (Fig. 6*A*). Moreover, the secretion of IFN-γ, TNF-α, IL-6, and IL-17 was higher in cells from the Roquin Tg mice than in those from WT mice, whereas IL-10 secretion was lower in cells from the Roquin Tg mice than in those from WT mice in a dose-dependent manner (Fig. 6*B*). These data show that enforced expression of Roquin in T cells increased their proliferation and cytokine production *in vitro*.

Altered Number of Lymphocytes in the Spleens of CIA Mice after Immunization—To evaluate the $CD4^+/CD28^+$ or $CD4^+/ICOS^+$ T cell populations, we isolated T cells from the spleens of mice with CIA using a $CD4^+$ T cell isolation kit. Expression of ICOS in $CD4^+$ T cells was lower in Roquin Tg mice than in WT mice, but CD28 expression in $CD4^+$ T cells was higher in Roquin Tg mice than in WT mice (Fig. 7*A*). Also, to evaluate the population of lymphocytes in Roquin Tg mice with CIA after immunization, we analyzed lymphocyte subsets in the spleen. The proportion of $Tbet^+CD44^{high}CD4^+$ T (Th1) cells was elevated in Roquin Tg mice compared with WT mice. However, that of $GATA3^+CD44^{high}CD4^+$ T (Th2) cells decreased in Roquin Tg mice compared with WT mice (Fig. 7*B*). Also, the proportion of $B220^+GL\text{-}7^+CD95^+$ (germinal center B) cells was higher in Roquin Tg mice, but that of $PD\text{-}1^{high}CXCR5^+CD4^+$ (Tfh) cells was unaffected (Fig. 7*C*). These results indicate that enforced expression of Roquin in the T cells of mice with CIA altered lymphocyte proportions and their expression of co-stimulatory molecules after immunization.

DISCUSSION

Previous studies of Roquin in autoimmunity suggested that its main role was suppression of autoimmune disease via ICOS down-regulation (23). However, our data suggest that enforced expression of Roquin in T cells accelerates induction of CIA.

Germ-line Roquin-deficient mice are perinatally lethal, so tissue-specific ablation of Roquin in T cells, B cells, and hematopoietic cells does not cause autoimmunity (24). For gain of

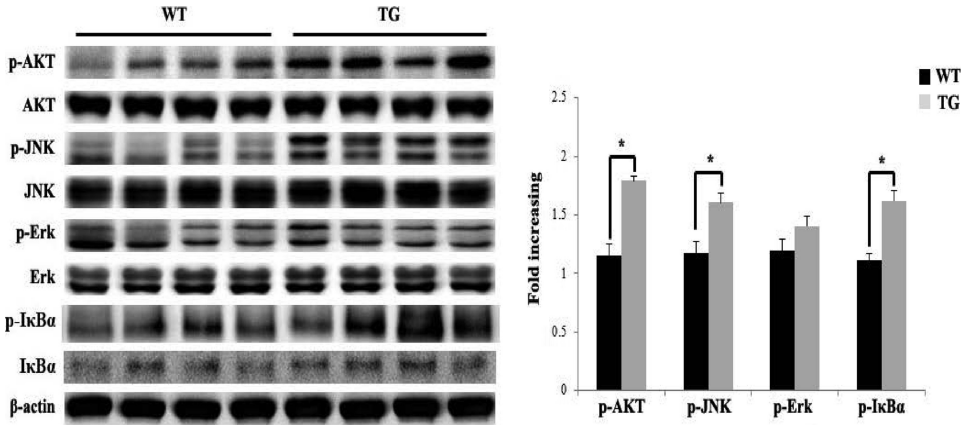

FIGURE 4. **Modulation of CD28 signaling by Roquin overexpression in mice.** Downstream signals of CD28 were evaluated by Western blot analysis on day 45 in spleen lysates from Roquin Tg and WT mice after immunization. The *right panel* shows data from the *left panel* that was graphed using ImageJ software. *Error bars* indicate mean ± S.E. of triplicates and are representative of three independent experiments. *, $p < 0.05$.

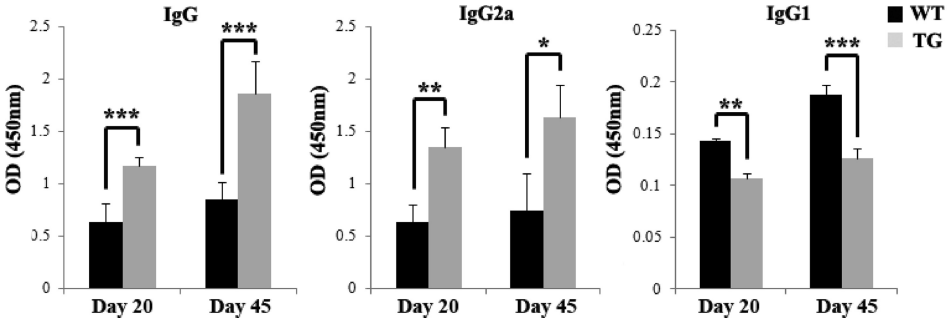

FIGURE 5. **Relative concentrations of CII-specific antibodies are altered in Roquin Tg mouse serum in collagen-induced arthritis.** Total IgG, IgG2a, and IgG1 anti-type II collagen antibody levels in sera from WT and Roquin Tg mice were determined on days 20 (diluted 1:5000) and 45 (diluted 1:20,000). *Black bars*, WT mice; *gray bars*, Roquin Tg mice. *Error bars* indicate mean ± S.E. of all immunized mice of each genotype ($n = 6$) and are expressed as the mean A_{450}. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

function via Roquin overexpression, we established Roquin Tg mice. We performed FACS analysis to determine the composition of the immune cell population. The Th1, Th2, germinal center B, and Tfh cell populations were unaffected under normal, non-immunized conditions. Although ICOS expression was down-regulated in the Roquin Tg mice, immune cell development was unaffected. Roquin Tg mice may not exhibit an immune defect because ICOS, another member of the CD28 family, is not expressed by naive T cells (13, 14).

Our data show that enforced expression of Roquin in T cells down-regulated ICOS and up-regulated CD28 expression in arthritis. Also, CD28 up-regulation and ICOS down-regulation were modulated by enforced expression of Roquin in EL-4 cells by anti-CD3/CD28 activation in our previous *in vitro* study (21). Both CD28 and ICOS are capable of stimulating the development of Th cells (25). However, ICOS is expressed only on T cells following activation, whereas CD28 is constitutively expressed. CD28-stimulated cells, but not ICOS-stimulated T cells, produce IL-2 (26). Thus, CD28 is important in the early stages of T cell activation, whereas ICOS has a major role in its maintenance. Also, ICOS provides a stimulatory co-signal to activated T cells rather than to naive T cells. Ablation of CD28 in mice induces resistance to CIA (12). Targeted deletion of ICOS or ICOS ligand in mice altered the number of Th cells and reduced disease activity in the CIA model of arthritis (7). Our results suggest that Roquin increases the development of arthritis, further suggesting that CD28 up-regulation by Roquin may have a greater effect than ICOS down-regulation in the initiation of T cell activation.

T cells stimulated by TNF-α, IFN-γ, and IL-17 become Th1 and Th17 helper cells, whereas those stimulated by IL-4 and IL-10 develop into Th2 helper cells (27, 28). TNF-α, IFN-γ, and IL-6 are proinflammatory cytokines produced by T cells and are involved in diverse biological processes such as final maturation of B cells into plasma cells and T cell activation. These cytokines seem to be of pivotal importance in arthritis (29–31). Our results show that serum TNF-α, IFN-γ, IL-6, and IL-17 levels were higher in Roquin Tg mice after immunization. In contrast, levels of IL-10, an anti-inflammatory cytokine (32), were lower in the serum of Roquin Tg mice after immunization. Therefore, these data suggest that Roquin Tg mice exhibit exacerbated development of CIA compared with WT mice.

CIA is a well known model of Th1-mediated autoimmune disease (33). This study demonstrated a novel role of Roquin function in the initiation and development of arthritis through its influence on Th1/Th2 differentiation and balance. $CD4^+$ T cells are classified into two major types: Th1 cells are primarily associated with cellular immunity and class switching to IgG2a, whereas Th2 cells are involved mainly in humoral immunity and class switching to IgG1 (34, 35). In the CIA model, immunization with CII generates a spectrum of antibodies with reactivities similar to those in human RA (4). Also, passive transfer of serum from CII-immunized animals or purified anti-CII antibodies can cause arthritis in syngeneic recipients (18, 36,

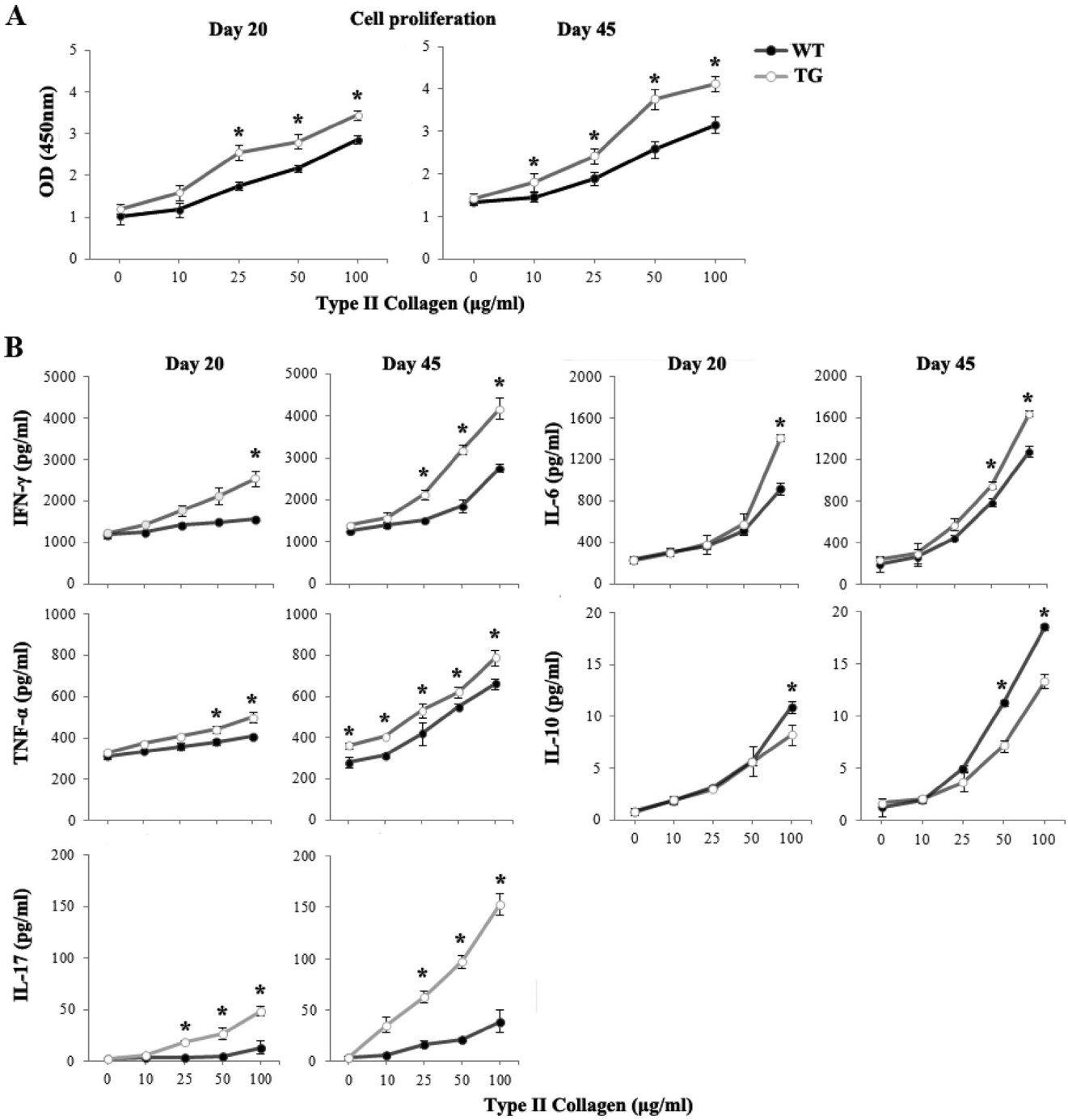

FIGURE 6. **Cell proliferation and cytokine production are increased by CII treatment.** *A*, spleen and draining lymph node cells were harvested on days 20 and 45 and cultured with graded CII concentrations for 96 h. The degree of cell proliferation was evaluated by a BrdU ELISA method after 96 h. *Error bars* indicate mean ± S.E. of triplicates and are representative of three independent experiments. *, $p < 0.05$. *B*, cytokine concentrations in culture supernatant were determined by ELISA after 72 h. *Solid symbols*, wild-type controls; *open symbols*, Roquin transgenic mice. *Error bars* represent the mean ± S.E. of triplicates and are representative of three independent experiments. *, $p < 0.05$.

37). Therefore, the production of IgG antibodies by Th1/Th2 cells plays a critical role in arthritis. Our data indicate increased IgG2a and decreased IgG1 levels in the serum of the Roquin Tg mice after CII immunization; thus, alterations in the levels of IgG2a and IgG1 may effectively alter the Th1/Th2 balance.

Our data demonstrate that Roquin may enhance Th1 development, and enforced expression of Roquin in T cells may result in an elevated Th1-type response after immunization. Th cell differentiation, a crucial step in the immune response, is the result of complex cellular and molecular regulation. Recent years have seen progress in understanding the transcriptional mechanisms of Th lineage commitment and effector cytokine expression. However, how these T cell-intrinsic mechanisms are regulated by environmental stimuli such as co-stimulation by antigen-presenting cells needs further definition. In the future, downstream signals of co-stimulatory receptors will be elucidated and likely found to operate via Roquin-regulated mechanisms. Thus, Roquin may be another "double-edged

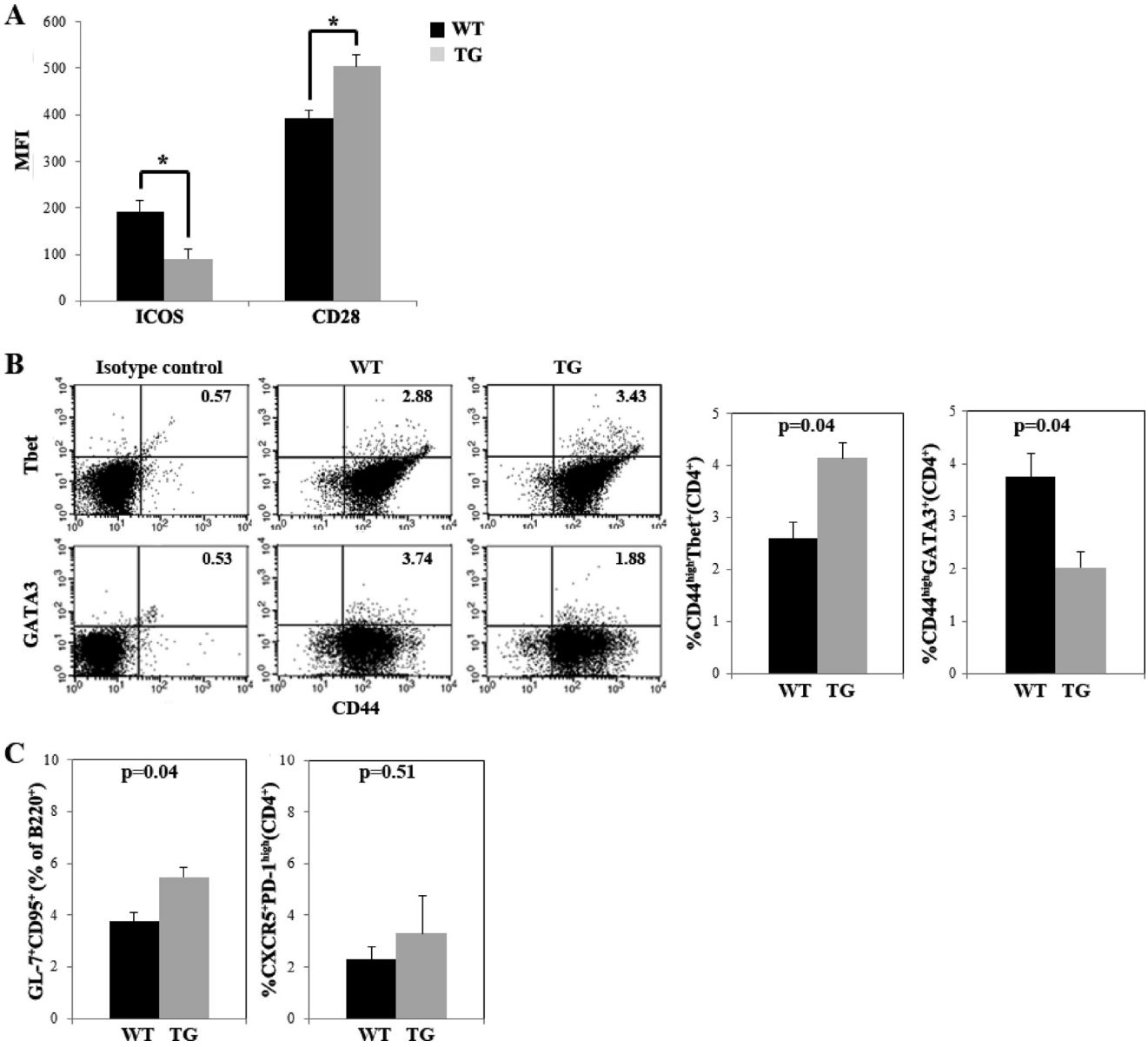

FIGURE 7. **Altered T cell populations in the spleen of collagen-induced arthritis mice after immunization.** *A*, populations of ICOS- and CD28-positive cells in $CD4^+$ T cells from WT and Roquin Tg mice on day 45 were determined by flow cytometry. Mean fluorescence intensity (*MFI*) values were measured and compared with those of WT mice. *Error bars* indicate the S.E. of triplicates. *, $p < 0.05$. *B*, representative flow cytometric plots (*left*) and graphic analysis (*right*) of $Tbet^+CD44^{high}CD4^+$ and $GATA3^+CD44^{high}CD4^+$ in WT and Roquin Tg mice on day 45. *C*, representative flow cytometric graphic analysis of $B220^+GL\text{-}7^+CD95^+$ and $PD\text{-}1^{high}CXCR5^+CD4^+$ cells in WT and Roquin Tg mice on day 45. *Black bars*, WT mice; *gray bars*, Roquin Tg mice. Data are representative of two independent experiments ($n \geq 4$ per group). *p* values are indicated on the graphs.

sword" for the immune system that can be turned from a requirement of autoreactivity to a regulator of Th1/Th2 differentiation and balance. In conclusion, elevated expression of Roquin in T cells may promote autoimmune diseases such as CIA.

REFERENCES

1. Kim, Y. O., Kim, H. J., Kim, S. K., Chung, J. H., and Hong, S. J. (2010) Association of the CD28/CTLA4/ICOS polymorphisms with susceptibility to rheumatoid arthritis. *Clin. Chem. Lab. Med.* **48,** 345–353
2. Zvaifler, N. J., and Firestein, G. S. (1994) Pannus and pannocytes. Alternative models of joint destruction in rheumatoid arthritis. *Arthritis Rheum.* **37,** 783–789
3. Brand, D. D., Latham, K. A., and Rosloniec, E. F. (2007) Collagen-induced arthritis. *Nat. Protoc.* **2,** 1269–1275
4. Holmdahl, R., Andersson, M., Goldschmidt, T. J., Gustafsson, K., Jansson, L., and Mo, J. A. (1990) Type II collagen autoimmunity in animals and provocations leading to arthritis. *Immunol. Rev.* **118,** 193–232
5. Holmdahl, R., Jansson, L., Larsson, A., and Jonsson, R. (1990) Arthritis in DBA/1 mice induced with passively transferred type II collagen immune serum. Immunohistopathology and serum levels of anti-type II collagen auto-antibodies. *Scand. J. Immunol.* **31,** 147–157
6. Seki, N., Sudo, Y., Yoshioka, T., Sugihara, S., Fujitsu, T., Sakuma, S., Ogawa, T., Hamaoka, T., Senoh, H., and Fujiwara, H. (1988) Type II collagen-induced murine arthritis. I. Induction and perpetuation of arthritis require synergy between humoral and cell-mediated immunity. *J. Immunol.* **140,** 1477–1484
7. Nurieva, R. I. (2005) Regulation of immune and autoimmune responses by ICOS-B7h interaction. *Clin. Immunol.* **115,** 19–25
8. June, C. H., Ledbetter, J. A., Linsley, P. S., and Thompson, C. B. (1990) Role of the CD28 receptor in T-cell activation. *Immunol. Today* **11,** 211–216
9. Ledbetter, J. A., Imboden, J. B., Schieven, G. L., Grosmaire, L. S., Rabino-

vitch, P. S., Lindsten, T., Thompson, C. B., and June, C. H. (1990) CD28 ligation in T-cell activation: evidence for two signal transduction pathways. *Blood* **75,** 1531–1539
10. Thompson, C. B., Lindsten, T., Ledbetter, J. A., Kunkel, S. L., Young, H. A., Emerson, S. G., Leiden, J. M., and June, C. H. (1989) CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 1333–1337
11. Shahinian, A., Pfeffer, K., Lee, K. P., Kündig, T. M., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, P. S., Thompson, C. B., and Mak, T. W. (1993) Differential T cell costimulatory requirements in CD28-deficient mice. *Science* **261,** 609–612
12. Tada, Y., Nagasawa, K., Ho, A., Morito, F., Ushiyama, O., Suzuki, N., Ohta, H., and Mak, T. W. (1999) CD28-deficient mice are highly resistant to collagen-induced arthritis. *J. Immunol.* **162,** 203–208
13. Hutloff, A., Dittrich, A. M., Beier, K. C., Eljaschewitsch, B., Kraft, R., Anagnostopoulos, I., and Kroczek, R. A. (1999) ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* **397,** 263–266
14. Yoshinaga, S. K., Whoriskey, J. S., Khare, S. D., Sarmiento, U., Guo, J., Horan, T., Shih, G., Zhang, M., Coccia, M. A., Kohno, T., Tafuri-Bladt, A., Brankow, D., Campbell, P., Chang, D., Chiu, L., Dai, T., Duncan, G., Elliott, G. S., Hui, A., McCabe, S. M., Scully, S., Shahinian, A., Shaklee, C. L., Van, G., Mak, T. W., and Senaldi, G. (1999) T-cell co-stimulation through B7RP-1 and ICOS. *Nature* **402,** 827–832
15. Dong, C., Juedes, A. E., Temann, U. A., Shresta, S., Allison, J. P., Ruddle, N. H., and Flavell, R. A. (2001) ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* **409,** 97–101
16. McAdam, A. J., Greenwald, R. J., Levin, M. A., Chernova, T., Malenkovich, N., Ling, V., Freeman, G. J., and Sharpe, A. H. (2001) ICOS is critical for CD40-mediated antibody class switching. *Nature* **409,** 102–105
17. Tafuri, A., Shahinian, A., Bladt, F., Yoshinaga, S. K., Jordana, M., Wakeham, A., Boucher, L. M., Bouchard, D., Chan, V. S., Duncan, G., Odermatt, B., Ho, A., Itie, A., Horan, T., Whoriskey, J. S., Pawson, T., Penninger, J. M., Ohashi, P. S., and Mak, T. W. (2001) ICOS is essential for effective T-helper-cell responses. *Nature* **409,** 105–109
18. Terato, K., Hasty, K. A., Reife, R. A., Cremer, M. A., Kang, A. H., and Stuart, J. M. (1992) Induction of arthritis with monoclonal antibodies to collagen. *J. Immunol.* **148,** 2103–2108
19. Lin, A. E., and Mak, T. W. (2007) The role of E3 ligases in autoimmunity and the regulation of autoreactive T cells. *Curr. Opin. Immunol.* **19,** 665–673
20. Vinuesa, C. G., Cook, M. C., Angelucci, C., Athanasopoulos, V., Rui, L., Hill, K. M., Yu, D., Domaschenz, H., Whittle, B., Lambe, T., Roberts, I. S., Copley, R. R., Bell, J. I., Cornall, R. J., and Goodnow, C. C. (2005) A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* **435,** 452–458
21. Kim, H. J., Ji, Y. R., Kim, M. O., Yu, D. H., Shin, M. J., Yuh, H. S., Bae, K. B., Park, S. J., Yi, J. K., Kim, N. R., Park, S. J., Yoon, D. H., Lee, W. H., Lee, S., and Ryoo, Z. Y. (2012) The role of Roquin overexpression in the modulation of signaling during *in vitro* and *ex vivo* T-cell activation. *Biochem. Biophys. Res. Commun.* **417,** 280–286
22. Rottman, J. B., Smith, T., Tonra, J. R., Ganley, K., Bloom, T., Silva, R., Pierce, B., Gutierrez-Ramos, J. C., Ozkaynak, E., and Coyle, A. J. (2001) The costimulatory molecule ICOS plays an important role in the immunopathogenesis of EAE. *Nat. Immunol.* **2,** 605–611
23. Yu, D., Tan, A. H., Hu, X., Athanasopoulos, V., Simpson, N., Silva, D. G., Hutloff, A., Giles, K. M., Leedman, P. J., Lam, K. P., Goodnow, C. C., and Vinuesa, C. G. (2007) Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. *Nature* **450,** 299–303
24. Bertossi, A., Aichinger, M., Sansonetti, P., Lech, M., Neff, F., Pal, M., Wunderlich, F. T., Anders, H. J., Klein, L., and Schmidt-Supprian, M. (2011) Loss of Roquin induces early death and immune deregulation but not autoimmunity. *J. Exp. Med.* **208,** 1749–1756
25. Carreno, B. M., and Collins, M. (2002) The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. *Annu. Rev. Immunol.* **20,** 29–53
26. Salomon, B., and Bluestone, J. A. (2001) Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. *Annu. Rev. Immunol.* **19,** 225–252
27. Choy, E. H., and Panayi, G. S. (2001) Cytokine pathways and joint inflammation in rheumatoid arthritis. *N. Engl. J. Med.* **344,** 907–916
28. Miossec, P. (2004) An update on the cytokine network in rheumatoid arthritis. *Curr. Opin. Rheumatol.* **16,** 218–222
29. Arend, W. P., and Dayer, J. M. (1995) Inhibition of the production and effects of interleukin-1 and tumor necrosis factor α in rheumatoid arthritis. *Arthritis Rheum.* **38,** 151–160
30. Kageyama, Y., Koide, Y., Yoshida, A., Uchijima, M., Arai, T., Miyamoto, S., Ozeki, T., Hiyoshi, M., Kushida, K., and Inoue, T. (1998) Reduced susceptibility to collagen-induced arthritis in mice deficient in IFN-γ receptor. *J. Immunol.* **161,** 1542–1548
31. Sasai, M., Saeki, Y., Ohshima, S., Nishioka, K., Mima, T., Tanaka, T., Katada, Y., Yoshizaki, K., Suemura, M., and Kishimoto, T. (1999) Delayed onset and reduced severity of collagen-induced arthritis in interleukin-6-deficient mice. *Arthritis Rheum.* **42,** 1635–1643
32. Asadullah, K., Sterry, W., and Volk, H. D. (2003) Interleukin-10 therapy—review of a new approach. *Pharmacol. Rev.* **55,** 241–269
33. Mauri, C., Williams, R. O., Walmsley, M., and Feldmann, M. (1996) Relationship between Th1/Th2 cytokine patterns and the arthritogenic response in collagen-induced arthritis. *Eur. J. Immunol.* **26,** 1511–1518
34. Mosmann, T. R., and Coffman, R. L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* **7,** 145–173
35. Paul, W. E., and Seder, R. A. (1994) Lymphocyte responses and cytokines. *Cell* **76,** 241–251
36. Stuart, J. M., and Dixon, F. J. (1983) Serum transfer of collagen-induced arthritis in mice. *J. Exp. Med.* **158,** 378–392
37. Holmdahl, R., Jansson, L., Larsson, E., Rubin, K., and Klareskog, L. (1986) Homologous type II collagen induces chronic and progressive arthritis in mice. *Arthritis Rheum.* **29,** 106–113